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Dephosphorylation of cdc2 on threonine 161 is required for cdc2 kinase inactivation and normal anaphase

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Exit from metaphase of the cell cycle requires inactivation of MPF, a stoichiometric complex between the cdc2 catalytic and the cyclin B regulatory subunits, as well as that of cyclin A–cdc2 kinase. Inactivation of both complexes depends on proteolytic degradation of the cyclin subunit, yet cyclin proteolysis is not sufficient to inactivate the H1 kinase activity of cdc2. Genetic evidence strongly suggests that type 1 phosphatase plays a key role in the metaphase–anaphase transition of the cell cycle. Here we report that inhibition of both type 1 and type 2A phosphatases by okadaic acid allows cyclin degradation to occur, but prevents cdc2 kinase inactivation. Complete inhibition of type 2A phosphatase alone is not sufficient to prevent cdc2 kinase inactivation following cyclin proteolysis. We show further that residue 161 of cdc2 is phosphorylated in active cyclin A or cyclin B complexes at metaphase, whilst unassociated cdc2 is not phosphorylated. Proteolysis of cyclin releases a free cdc2 subunit, which subsequently undergoes dephosphorylation and then migrates more slowly than its Thr161 phosphorylated counterpart in Laemmli gels. Removal of phosphothreonine 161 requires cyclin proteolysis. However, it does not occur even after cyclin proteolysis, when both type 1 and type 2A phosphatases are inhibited. We conclude that both cyclin degradation and dephosphorylation of Thr161 on cdc2, catalysed at least in part by type 1 phosphatase, are required to inactivate either cyclin B– or cyclin A–cdc2 kinases and thus for cells to exit from M phase.

Key words: cdc2 kinase/cell cycle/type 1 phosphatase/type 2A phosphatase

Introduction

Exit from M phase of the cell cycle requires inactivation of MPF, a stoichiometric complex between the cdc2 catalytic and the cyclin B regulatory subunits (for reviews see Dorée, 1990; Nurse, 1990). This inactivation depends on proteolytic degradation of the cyclin subunit (Murray *et al.*, 1989; Ghiara *et al.*, 1991). Another 'mitotic' cyclin, cyclin A, which differs from cyclin B by specific conserved motifs in both the 'cyclin box' and the 'destruction box' (for review see Nugent *et al.*, 1991), also forms complexes with cdc2 and in addition with at least one protein related to but distinct from cdc2, cdk2 (Pines and Hunter, 1990; Paris *et al.*, 1991; Tsai *et al.*, 1991). Proteolytic degradation of cyclin A is also required for cells to exit from M phase (Luca *et al.*, 1991). Degradation of both cyclin A and B is mediated by a ubiquitin-dependent process which requires integrity of the 'destruction box' (Glotzer *et al*, 1991; Hershko *et al.*, 1991; Lorca *et al.*, 1991a). In contrast with the well documented requirement of cyclin proteolysis for cdc2 kinase inactivation, there is no direct evidence that cyclin proteolysis is sufficient to inactivate cdc2 kinase and exit from M phase.

In vitro experiments have shown that cyclin B–cdc2 kinase can be inactivated alternatively by treating partially purified complexes with type 2A phosphatase or a related protein phosphatase called INH (Gould *et al.*, 1991; Lee *et al.*, 1991). In these experiments phosphatases probably removed phosphate from cdc2 on residue Thr161 or its homologue Thr167 in fission yeast because Thr161/167 was found to be the only consistently phosphorylated residue in active cyclin B–cdc2 complexes (Gould *et al.*, 1991; Krek and Nigg, 1991; Solomon *et al.*, 1992).

Whether the dephosphorylation on Thr161 is essential for cdc2 kinase to undergo inactivation had not been investigated previously, although circumstantial evidence exists to support the view that cyclin-free cdc2 subunit may have protein kinase activity in some cases (see Discussion). The aim of this work was to investigate whether dephosphorylation of Thr161 actually accompanies cyclin degradation and whether it is required for cdc2 kinase inactivation. We conclude that both cyclin degradation and dephosphorylation of the Thr161 residue on cdc2, catalysed at least in part by type 1 phosphatase, are required to inactivate both cyclin B– and cyclin A–cdc2 kinases and thus for cells to exit from M phase. This completes the picture of how cell cycle dependent phosphorylation of cdc2 at multiple sites is coordinated with its association with cyclins.

Results

A low mobility form of cdc2 is produced when cyclin proteolysis is induced in Xenopus extracts

In order to dissect the mechanism of cdc2 kinase inactivation, we first used extracts prepared from unfertilized *Xenopus* eggs. Such eggs are blocked at metaphase II due to a cytostatic factor (CSF) which prevents cyclin degradation (Masui and Markert, 1971; Sagata *et al.*, 1988) and is itself inactivated by a Ca^{2+}–calmodulin-dependent process (Lorca *et al.*, 1991b).

We first monitored changes in the electrophoretic mobility of cdc2 that accompany the metaphase II to interphase transition induced by Ca^{2+}. Cdc2 was adsorbed on $p13^{suc1}$ beads and analysed by Western blotting using an affinity-purified antibody directed against the N-terminal 12 amino acid peptide of cdc2 (hereafter referred to as NMPF). This antibody does not cross-react with cdk2/Eg1 (Clarke *et al.*, 1992; A.Devault, D.Fesquet, J.-C.Cavadore,

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A.M.Garrigues, J.-C.Labbé, A.Picard, M.Philippe and M.Dorée, submitted), the inactivation of which was not investigated in this paper. Indeed cdk2 does not bind cyclin B and only poorly binds cyclin A, and contributes to only a small fraction of the H1 histone kinase activity in early frog extracts, even upon addition of excess recombinant cyclin A (Minshull *et al.*, 1990; Devault *et al.*, 1991; Fang and Newport, 1991; Roy *et al.*, 1991; Clarke *et al.*, 1992). Two immunoreactive bands of apparent M_r 34 000 and 35 000 Da (p34 and p35) were detected in CSF extracts under our electrophoretic conditions (Figure 1A, lane 1). Neither of these bands contained phosphotyrosine (Figure 1B). When recombinant cyclin A (or B, see below Figure 3) was added to CSF extracts in sufficient amount to saturate cdc2, the lower mobility component was rapidly converted to the higher mobility component (Figure 1A, lane 2) and H1 histone kinase activity increased in the extracts (Figure 1C). A similar downshift of cdc2 was observed by others upon cyclin addition to clam or frog extracts (Luca *et al.*, 1991; Clarke *et al.*, 1992). This indicated that p35 actually corresponds to free monomeric cdc2 and p34 to cyclin associated cdc2. We confirmed this result by showing that antibodies directed against *Xenopus* B1 and B2 cyclins immunoprecipitate p34 and not p35 from CSF extracts (Figure 1D).

Then 0.4 mM Ca^{2+} was added to inactivate CSF. After 30 min both endogenous cyclins and the added cyclin A had undergone proteolysis (data not shown), cdc2 had shifted to a lower mobility form migrating exactly as the p35 component found in CSF extracts (Figure 1A, lane 3) and the H1 kinase activity had dropped (Figure 1C). No phosphotyrosine was detected in this low mobility form of cdc2 (data not shown). Again, addition of recombinant cyclin A converted cdc2 from its p35 to p34 form (Figure 1A, lane 4) and this was associated with a marked increase in H1 kinase activity (Figure 1C). These results suggested that some process modifies cdc2 when the associated cyclin subunit is proteolysed in CSF extracts.

In order to investigate whether this is the case during the regular cell cycle, we then used extracts of parthenogenetically activated *Xenopus* eggs blocked at interphase by cycloheximide treatment. Such extracts do not contain immunodetectable cyclins (data not shown). To convert this 'interphase' extract to a 'mitotic' extract, purified starfish cyclin B–cdc2 kinase was added (Tuomikoski *et al.*, 1989; Verde *et al.*, 1990; Pypaerts *et al.*, 1991). After 30 min starfish cyclin B had readily undergone proteolysis, in agreement with previous results (Felix *et al.*, 1990b). Moreover, the apparent M_r of starfish cdc2 had shifted from a 34 kDa to a 35 kDa form and the H1 kinase activity had dropped (Figure 2). These changes are similar to those observed after CSF inactivation in extracts prepared from metaphase II arrested eggs.

Cyclin degradation is required to produce the low mobility form of cdc2

The above results indicated that cyclin associated cdc2 shifts to a slow migrating form when a mitotic extract, with or without CSF, is converted to an interphase extract by triggering cyclin degradation. To determine whether modification of cdc2 mobility was merely an indirect consequence of changing the overall properties of the extracts from a 'mitotic' to an 'interphasic' state, or alternatively

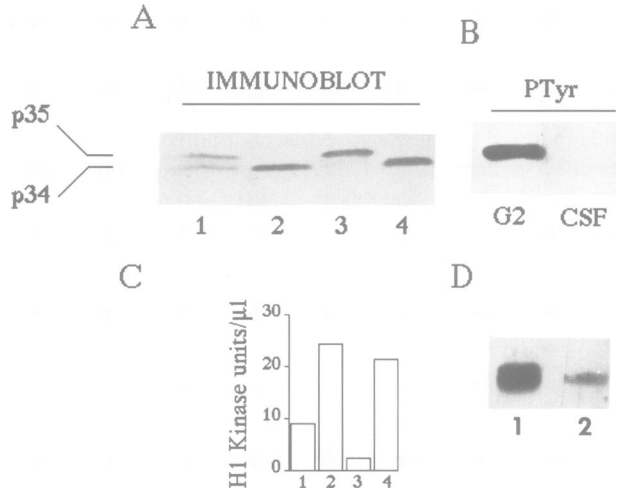

Fig. 1. A low mobility form of cdc2 is produced when cyclin proteolysis is induced in CSF extracts. **(A)** Changes in electrophoretic mobility of cdc2 (recovered from extracts by affinity on $p13^{suc1}$ beads) as detected by immunoblotting with the NMPF antibody. 1, CSF extract; 2, recombinant cyclin A was added to saturate cdc2; 3, 0.4 mM Ca^{2+} was added (after cyclin A addition) to trigger cyclin degradation; 4, recombinant cyclin A was added again (after the first round of cyclin degradation). **(B)** CSF extracts and extracts prepared from G_2-arrested oocytes (control) were Western blotted and cdc2 immunodetected with specific antibodies against phosphotyrosine. **(C)** Same experiment as in (A) (same numbering), but aliquots were taken and used for determination of H1 kinase activities. **(D)** Cyclins B1 and B2 were immunoprecipitated from a CSF extract using a mixture of antibodies against either cyclin. The immunoprecipitated material was analysed by Western blotting with the NMPF antibody directed against cdc2 (lane 2). Lane 1 is a control run on the same gel showing position of cdc2 (p34 and p35) in the whole CSF extract.

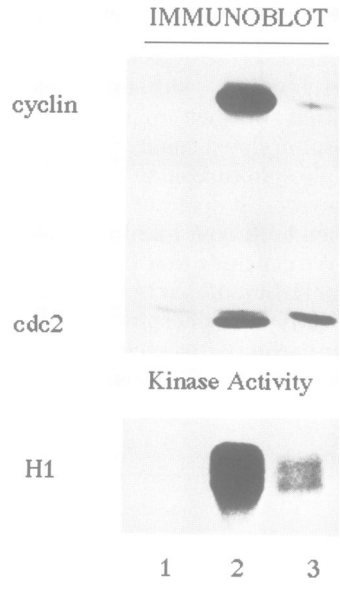

Fig. 2. A low mobility form of cdc2 is produced after cyclin proteolysis in interphase extracts. Highly purified starfish cyclin B–cdc2 kinase was added (50 units/µl) to a *Xenopus* extract prepared from parthenogenetically activated eggs blocked at interphase by cycloheximide. Aliquots were taken either immediately after kinase addition (lane 2) or 30 min later (lane 3) and processed either for determination of starfish cyclin B and cdc2 (both the *Xenopus* and the starfish one) by Western blot analysis with the corresponding antibodies or for measurement of H1 kinase activities. Lane 1 is a control, without the addition of starfish cdc2 kinase.

whether it absolutely required degradation of the associated cyclin subunit, a C-terminus tagged recombinant starfish cyclin B was added to a CSF extract. Due to internal initiation of translation in *Escherichia coli*, the recombinant cyclin B comprised both the full-length protein and a truncated form lacking the destruction box (Δ72). As observed previously for cyclin A, addition of cyclin B converted the low mobility form of cdc2 into its high mobility form (Figure 3, lane 2). Interestingly, this conversion was rapid (<5 min) and no transient tyrosine phosphorylated form of cdc2 was detected in such conditions (data not shown). 0.4 mM Ca^{2+} was then added to inactivate CSF, after 1 h the truncated cyclin B that escaped proteolysis was recovered together with its associated cdc2 subunit by affinity chromatography on human serum albumin (HSA) beads, whilst the remaining cyclin-free cdc2 was recovered by affinity on $p13^{suc1}$ beads. The low mobility form of cdc2 was detected only on $p13^{suc1}$ beads (Figure 3, lane 4) and the high mobility form only on HSA beads (Figure 3, lane 3). Thus in the same extract cdc2 was either converted or not converted to the low mobility form depending on whether it was still associated or not to a cyclin subunit. This indicates that conversion of cdc2 from the high to the low mobility form requires cyclin proteolysis.

Inhibition of okadaic acid sensitive phosphatases prevents both the conversion of cdc2 from its high to low mobility form and the drop of H1 kinase activity after cyclin degradation

CSF and interphase *Xenopus* extracts contain both type 1 and type 2A phosphatases. The concentrations of okadaic acid (OA) required to mainly inhibit type 2A phosphatase alone or both type 2A and type 1 phosphatases in these concentrated extracts have been determined previously, using the α- and β-labelled subunits of phosphorylase kinase as substrates for type 2A and type 1 phosphatases, respectively (Felix *et al.*, 1990a). An almost complete inhibition of type 2A phosphatase was obtained by adding 0.9 μM OA, whereas only a 50% inhibition of type 1 phosphatase was measured at this concentration. When OA concentration was raised to 2.5 μM, both type 2A and type 1 phosphatases were completely inhibited. We repeated these experiments and confirmed these results (data not shown).

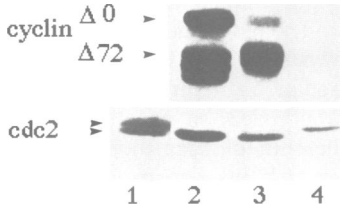

Fig. 3. Cyclin degradation is required to produce the low mobility form of cdc2. A tagged recombinant starfish cyclin B (comprising both Δ0, the full-length protein, and Δ72, a truncated form lacking the destruction box) was added to a CSF extract. Then Ca^{2+} (0.4 mM) was added to induce cyclin degradation. Samples were taken either before (lane 2) or 1 h after Ca^{2+} addition (lanes 3 and 4) and treated first with HSA beads to quantitatively recover the tagged cyclin (lanes 2 and 3) then with $p13^{suc1}$ beads to recover residual cdc2 (lane 4). Materials retained on HSA and $p13^{suc1}$ beads were eluted and analysed by Western blotting using either NMPF antibodies (for cdc2) or specific antibodies against starfish cyclin B. Lane 1 is a control without the addition of starfish cyclin B (the material retained on $p13^{suc1}$ beads was analysed).

Preliminary experiments (data not shown) indicated that 2.5 μM OA could prevent the shift of cdc2 from its high to its low mobility form and the concomitant drop of H1 kinase activity, which follows inactivation of CSF by Ca^{2+}, without preventing cyclin degradation. In order to improve quantification of the H1 kinase activity and as a first step towards a large scale preparation of the active cdc2 kinase monomer, we repeated these experiments after adding highly purified starfish cyclin B−cdc2 kinase, which raised the H1 kinase activity from 10 to ~100 units/μl in CSF extracts. OA was added to final concentrations of either 0.9 or 2.5 μM, then 0.4 mM Ca^{2+} was added to inactivate CSF and trigger cyclin degradation. As shown in Figure 4, cyclin readily underwent degradation at both OA concentrations (upper panel). However, cdc2 was converted to its lower mobility form and cdc2 kinase was inactivated only in the presence of the lower concentration of OA (middle and lower panels). When both type 2A and type 1 phosphatases were blocked in the extracts by OA at the higher concentration, no shift of cdc2 was observed and no significant decrease of cdc2 kinase activity was detected even after the cyclin subunit had undergone complete proteolysis.

We next used interphase extracts containing either 0.9 or 2.5 μM OA and H1 kinase activity was generated by adding 60 nM recombinant starfish cyclin B (i.e. a cyclin comprising both the full-length and a truncated protein lacking the destruction box). As confirmed by monitoring the degradation of tracer cyclin B (Figure 5, upper panel), proteolysis was turned on at both OA concentrations. After 1 h the fraction of tagged cyclin B which escaped proteolysis was quantitatively recovered together with its associated cdc2 subunit on HSA beads and the remaining cdc2, free of any association with cyclin, on $p13^{suc1}$ beads. As expected, only

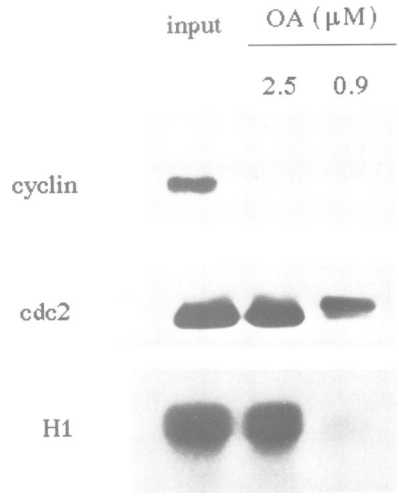

Fig. 4. Simultaneous inhibition of type 1 and type 2A phosphatases by OA prevents both the conversion of cdc2 from its high to low mobility form and H1 kinase inactivation following induction of cyclin degradation by Ca^{2+} in CSF extracts. Highly purified starfish cdc2 kinase was added to CSF extracts containing either 0.9 or 2.5 μM OA, raising H1 kinase activity from 10 to ~100 units/μl. Then Ca^{2+} (0.4 mM) was added to some extracts in order to activate cyclin degradation. Aliquots were taken 30 min after Ca^{2+} addition, purified by affinity on $p13^{suc1}$ beads and the retained material was either analysed by immunoblotting to determine the amount of residual starfish cyclin B **(upper panel)** and the mobility of cdc2 **(middle panel)** or assayed for H1 kinase activity **(lower panel)** as compared to the input material.

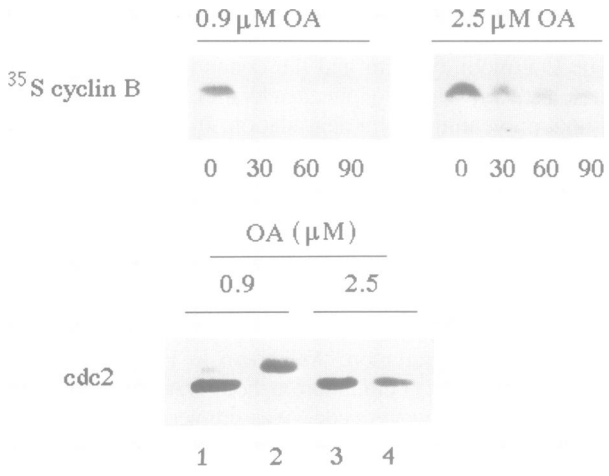

Fig. 5. Inhibition of both type 1 and 2A phosphatases prevents conversion of cdc2 from its high to low mobility form following cyclin degradation in interphase extracts. A tagged recombinant starfish cyclin B (comprising both the full-length protein and a truncated form lacking the destruction box) was added to extracts prepared at interphase, 20 min after parthenogenetic activation of *Xenopus* eggs, in order to generate the cdc2 kinase activity required to turn on the cyclin degradation pathway. The interphase extracts were also complemented with OA (0.9 or 2.5 μM). **Upper panel.** ^{35}S-labelled human cyclin B, translated (without internal initiation) in the reticulocyte system, was added simultaneously with recombinant starfish cyclin B to monitor kinetics of cyclin degradation in the extract. Samples were taken at the indicated times (min) and processed for determination of residual [^{35}S]cyclin B by autoradiography. **Lower panel.** Same experiment as in the upper panel, but aliquots were taken either 10 min (lanes 1 and 3, before cyclin degradation) or 40 min after recombinant cyclin addition (lanes 2 and 4, after full length cyclin degradation). After affinity purification (see below), they were analysed by immunoblotting with NMPF antibodies for electrophoretic mobility of cdc2. Lanes 1 and 3, material retained on HSA beads; lanes 2 and 4, material retained on p13^{suc1} beads after depletion on HSA beads of truncated cyclin-containing complexes.

the high mobility form of cdc2 was detected in the material eluted from HSA beads at both OA concentrations (lower panel, lanes 1 and 3). In contrast, cyclin-free cdc2 had different mobilities, depending on OA concentration. Addition of 0.9 μM OA allowed (lane 2) the conversion of the high to low mobility form of cdc2 following destruction of the associated cyclin subunit, but 2.5 μM OA prevented it (lane 4).

The above results could be interpreted in either of two ways. Either blocking type 1 phosphatase alone in *Xenopus* extracts was sufficient or blocking both type 1 and type 2A phosphatases was required to prevent cdc2 from shifting after cyclin destruction. In order to examine the first possibility we tested the effect of inhibitor 1, a reported specific inhibitor of type 1 phosphatase. A CSF extract was pre-incubated for 5 min with 1 μM of the active thiophosphorylated peptide of inhibitor 1 (residues 9–41), then 0.4 mM Ca^{2+} was added. 0.5 μM of the inhibitor was added every 10 min during the 1 h incubation, in case it was degraded or dephosphorylated in the extracts. As shown in Figure 6, inhibitor 1 prevented neither the shift of cdc2 nor the inactivation of H1 kinase after cyclin degradation. This suggested that both type 1 and type 2A phosphatases must be blocked simultaneously to prevent cdc2 from shifting from the high to the low mobility form in frog extracts.

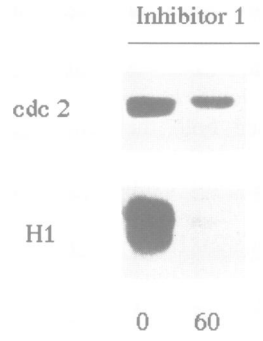

Fig. 6. Inhibitor 1 prevents neither conversion of cdc2 from its high to low mobility form nor H1 kinase inactivation following cyclin degradation induced by Ca^{2+} in CSF extracts. A CSF extract was incubated with inhibitor 1 (1 μM at first, then 0.5 μM every 10 min), then Ca^{2+} (0.4 mM) was added to induce cyclin degradation. Samples were taken either before (0) or 60 min after Ca^{2+} addition and either used for determination of cdc2 electrophoretic mobility by immunoblotting (top) or for determination of H1 kinase activities (bottom).

Dephosphorylation of Thr161 accompanies the shift of cdc2 from a high to a low mobility form on exit from M phase

The above results showed that a dephosphorylation event was required for cdc2 to shift to its low mobility form after cyclin proteolysis, when it loses its H1 histone kinase activity. Dephosphorylation could occur at the level of cdc2 itself. To investigate this possibility, we repeated the first step of the experiment depicted in Figure 1, but this time recombinant cyclin A was added to extracts containing [γ-^{32}P]ATP. Then cyclin A-containing complexes were recovered on anti-cyclin A beads and the immunoaffinity-purified materials were analysed. Radioactivity was recovered in both cyclin A and cdc2 in immunoprecipitates (Figure 7, left panel, lane 1). Phosphoamino acid analysis demonstrated the presence of phosphothreonine in cdc2 (Figure 7, bottom right panel) and phosphoserine in cyclin A (data not shown). No radioactivity was recovered with monomeric cdc2 immunoprecipitated by antibodies (NMPF and PSTAIR) inefficient at detecting cdc2 in cyclin–cdc2 complexes (lane 4) (Pines and Hunter, 1990). In contrast radioactivity was recovered with cdc2 when cyclin–cdc2 complexes were first dissociated by boiling in the presence of SDS, before being immunoprecipitated with the same antibodies (lane 3). The radioactivity of cyclin A-associated cdc2 disappeared when Ca^{2+} was added to CSF extracts to trigger cyclin degradation (Figure 7, top right panel). Thus dephosphorylation of cdc2 on a threonine residue was associated with the shift of cdc2 mobility which follows cyclin degradation and this dephosphorylation could be suppressed by OA. However, it is unusual that dephosphorylation of a protein decreases its electrophoretic mobility, although phosphorylated human cdk2 was recently reported to migrate faster than its unphosphorylated counterpart (Pines and Hunter, 1992). Most commonly, a decreased mobility is rather typical of a phosphorylation event.

In order to confirm this unexpected result, *Xenopus* oocytes were pre-loaded with ^{32}P and induced to mature with progesterone. When they had arrested at metaphase II, CSF extracts were prepared and cdc2 was recovered by affinity chromatography on p13^{suc1} beads. As expected, the

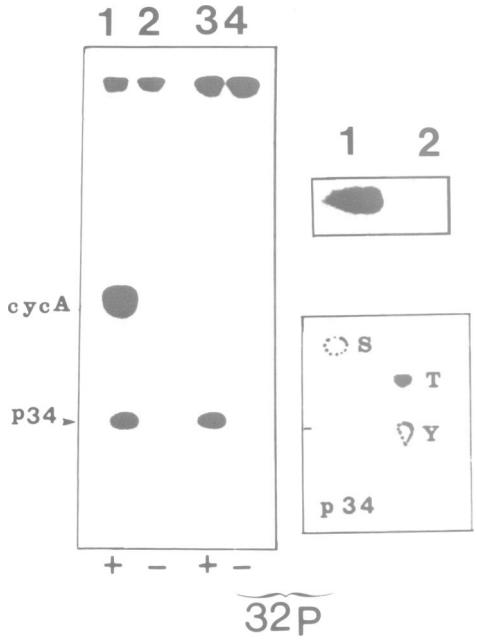

Fig. 7. *In vitro* ^{32}P-labelling of cyclin associated cdc2 on a threonine residue in *Xenopus* extracts. **Left panel.** Cyclin A (150 nM) was added (+, lanes 1 and 3) or not (−, lanes 2 and 4) to a frog extract in the presence of [γ-^{32}P]ATP (6 mCi/ml). After 30 min either cyclin A-containing complexes were recovered on anti-cyclin A beads (lanes 1 and 2, control) or free cdc2 was immunoprecipitated with a 1/1 mixture of the PSTAIR and NMPF sera, after heating in the presence of 1% SDS (in order to dissociate multimolecular complexes) and a 10-fold dilution with RIPA buffer (lanes 3 and 4). Finally the immunoaffinity-purified materials were analysed by SDS−PAGE and autoradiography. **Right panel** (bottom): Phosphoamino acid analysis of [γ-^{32}P]cdc2 eluted from lane 1; right panel (top): Same experiment as in the left panel, but Ca^{2+} was added to the extract (in the continuous presence of [γ-^{32}P]ATP) to turn on cyclin degradation. Lane 1, an aliquot was taken before cyclin degradation and immunoprecipitated with anti-cyclin A antibodies, the autoradiograph shows ^{32}P-labelled cdc2; lane 2, an aliquot of extract was taken after cyclin degradation and immunoprecipitated with a 1/1 mixture of PSTAIR and NMPF serums, no label is associated with cdc2.

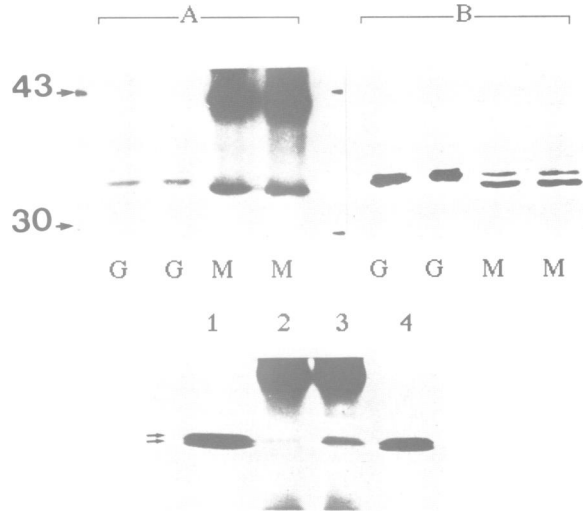

Fig. 8. *In vivo* ^{32}P-labelling of cyclin associated cdc2 in unfertilized *Xenopus* eggs arrested at second meiotic metaphase. **Upper panel.** *Xenopus* oocytes were preloaded with [^{32}P]orthophosphate (1 mCi/ml) and induced to mature with progesterone. Extracts were prepared from G_2-arrested (G) or metaphase II-arrested oocytes (M) then the material retained on p13^{suc1} beads was eluted, run on SDS−polyacrylamide gels followed by Western blotting and analysed either by autoradiography (A) or by immunodetection of cdc2 by NMPF antibodies (B). Duplicate samples were run on the same gel. Arrows point to position of markers (ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa). **Lower panel.** A CSF extract was either treated first with p13^{suc1} beads (1) then the depleted extract immunoprecipitated with the PSTAIR antibody (2) or conversely the extract was immunoprecipitated first with the PSTAIR antibody (3) and the corresponding supernatant was treated with p13^{suc1} beads (4). Precipitated materials were analysed by immunoblotting with NMPF antibodies.

NMPF antibody recognized two immunoreactive bands in such extracts, p34 and p35, (Figure 8, upper panel, lanes M). However, only p34 was found to contain ^{32}P. We showed in a previous section that p34 corresponds to cdc2 associated to cyclin and p35 to free cdc2. This was confirmed by showing that the PSTAIR antibody, which does not recognize cdc2 efficiently in cyclin−cdc2 complexes (Pines and Hunter, 1990), readily immunoprecipitated p35 but failed to immunoprecipitate p34 in CSF extracts (Figure 8, lower panel, lane 3). Moreover PSTAIR immunoprecipitates failed to phosphorylate significantly H1 histone in contrast to p13^{suc1} precipitates (data not shown). These results are consistent with the view that threonine phosphorylated cdc2 migrates faster than the dephosphorylated and cyclin-free cdc2 subunit.

Threonine dephosphorylation of cdc2 after cyclin proteolysis could be explained in either of two ways. Either phosphothreonine does not turn over in cyclin−cdc2 complexes and destruction of cyclin is required for the phosphorylated residue to become accessible to the OA-sensitive phosphatase(s), which dephosphorylate(s) cdc2; or the OA-sensitive phosphatase(s) can dephosphorylate even in association with cyclin cdc2, but the antagonizing kinase which phosphorylates cdc2 on the unique threonine residue is specific for cyclin−cdc2 and cannot phosphorylate the unassociated subunit. In the last case, activity of the non-antagonized phosphatase(s) after cyclin proteolysis would result in a net dephosphorylation of cdc2. To investigate which of these hypotheses was correct, cyclin A was added to a CSF extract containing [γ-^{32}P]ATP then cyclin A complexes containing threonine phosphorylated cdc2 were recovered on anti-cyclin A beads and transferred into a CSF extract free of labelled ATP, either in the absence or the presence of 2.5 μM OA. Samples were taken at various times after transfer and analysed by autoradiography for cdc2 phosphorylation. As shown in Figure 9A, radioactivity had almost disappeared from cdc2 after 10 min in the absence of OA. In contrast, no significant decay of cdc2 associated radioactivity was detected, even after 20 min incubation in CSF extracts when both type 2A and type 1 phosphatases were completely blocked with 2.5 μM OA. Conversely, the stable cyclin A−cdc2 complex, which formed in CSF extracts upon addition of recombinant cyclin A in the absence of radioactivity, could be subsequently labelled by adding [γ-^{32}P]ATP to the extracts (Figure 9B). We conclude that a unique phosphothreonine residue of cdc2 turns over in active cyclin−cdc2 complexes but disappears only upon cyclin proteolysis.

What could be this phosphothreonine residue? Cyclin B−cdc2 kinase has been shown to be phosphorylated on a single residue, Thr167 in fission yeast (Gould *et al.*, 1991) and its homologue Thr161 in higher eukaryotes (Krek and Nigg, 1991). This was probably the site which undergoes

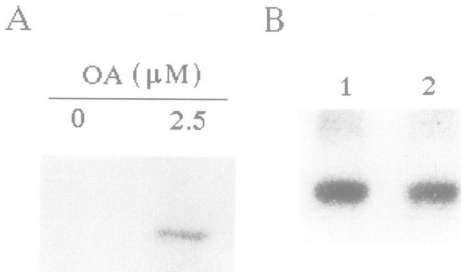

Fig. 9. Phosphorylation of cdc2 on a threonine residue (Thr161) turns over at metaphase in cyclin−cdc2 complexes and is prevented from turning over by 2.5 μM OA. **(A)** Cyclin A (100 nM) was added to a CSF extract containing [γ-^{32}P]ATP (6 mCi/ml) and after 30 min cyclin A complexes (containing threonine phosphorylated cdc2) were recovered by immunoprecipitation and transferred into a CSF extract free of labelled ATP, either in the absence (0) or presence of 2.5 μM OA. Samples were taken 10 min after transfer to the OA-free extract (left) or 20 min after transfer to the OA-containing extract (right), and the anti-cylin A immunoprecipitates eluted and analysed by SDS−PAGE and autoradiography: only cdc2 is shown. **(B)** Cyclin A (100 nM) was added to a CSF extract with (1) or without (2) [γ-^{32}P]ATP (6 mCi/ml). In the first case (1) cyclin A-containing complexes were left to incubate for 1 h in the extract in the continuous presence of [γ-^{32}P]ATP then recovered by immunoprecipitation. In the second case (2) cyclin A-containing complexes were left to incubate for 30 min in the extract in the absence of [γ-^{32}P]ATP, then [γ-^{32}P]ATP was added to the same specific activity as in (1). After 30 min cyclin A-containing complexes were recovered by immunoprecipitation, eluted and analysed by SDS−PAGE and autoradiography: only cdc2 is shown.

dephosphorylation upon cyclin degradation, but it was not known whether cdc2 is also phosphorylated on this residue in cyclin A−cdc2 complexes. This prompted us to map the phosphorylated site, which seems to confer on cdc2 its surprising electrophoretic mobility, in both cyclin A− and cyclin B−cdc2 complexes.

Xenopus cdc2 contains four tryptophan residues at positions 168, 188, 228 and 244. When cyclin A-associated and radiolabelled cdc2 was degraded by *N*-chlorosuccinimide, three ^{32}P-labelled fragments recognized by Western blotting with the NMPF antibody were produced, in addition to undegraded cdc2 (Figure 10, panel A). Since the three fragments contained the N-terminal end of cdc2, the shorter one could be either the 1−166 or the 1−188 fragment.

The radioactive residue was recovered in a fragment migrating with an apparent M_r of 18 kDa following degradation of cdc2 by cyanogen bromide (Figure 10, panel B). Since this fragment was not recognized by the PSTAIR antibody (data not shown), the possibility that phosphothreonine might be between residues 1 and 71 was eliminated. Indeed the PSTAIR peptide is localized between two methionine residues located at positions 32 and 71. Hence, phosphothreonine was located between residues 72 and 188.

Only four threonine residues are found in this fragment, at positions 102, 161, 166 and 183. To distinguish between these possibilities, tryptic fragments were left unmodified or treated with either the V8 protease alone or both the V8 protease and *N*-chlorosuccinimide. The resulting material was then analysed by TLE and autoradiographed. Treatment with V8 was found to change mobility of the labelled fragment (Figure 10, panel C). This excluded the possibility that phosphothreonine was at residue 102 because the tryptic peptide spanning this residue does not contain glutamic acid.

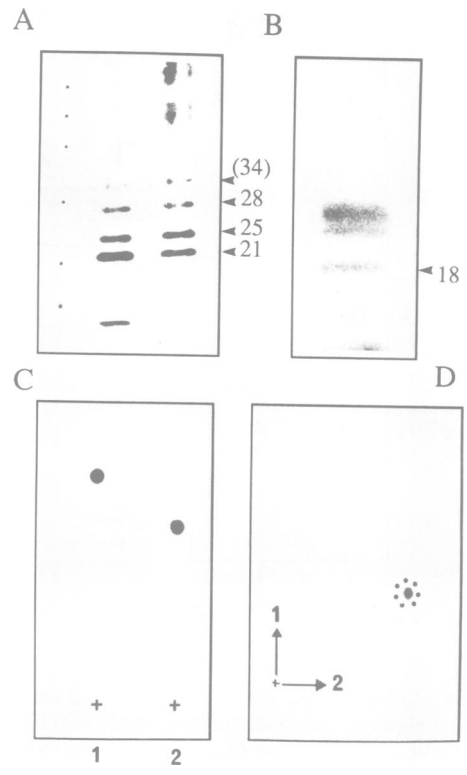

Fig. 10. Peptide mapping of phosphothreonine in cyclin A associated and ^{32}P-labelled cdc2. **(A)** Analysis of the degradation products of cdc2 by *N*-chlorosuccinimide. The degradation products were separated by SDS−PAGE and transferred to a nitrocellulose sheet, which was either submitted to autoradiography (left) or probed with the NMPF antibody (right). Arrowheads point to undegraded cdc2 (34 kDa) and NCS fragments containing an intact N-terminus and thus recognized by the NMPF antibody. **(B)** Analysis of the degradation products of cdc2 by cyanogen bromide. The degradation products were separated by SDS−PAGE and submitted to autoradiography. The arrowhead points to the smaller fragment retained on the 12% polyacrylamide gel (very small fragments were also produced but they migrated as fast as the tracking dye and were lost from the gel). **(C)** Compared mobilities of ^{32}P-labelled cdc2 tryptic fragments not treated (1) or treated (2) with the V8 protease. Samples were loaded at positions indicated by crosses. Mono-dimensional electrophoresis was run at pH 1.9. **(D)** The ^{32}P-labelled fragment resulting from sequential digestion of the whole cyclin A associated cdc2 by trypsin and the V8 protease co-migrates with the synthetic Val-Tyr-Thr(P)-His-Glu phosphopeptide. The sample was loaded at the position indicated by the cross. First dimension TLE (1) and second dimension TLC (2). The dashed circle around the radiolabelled spot indicates the position of the synthetic phosphopeptide, as it was localized with a ninhydrin-containing spray.

Finally we synthetized the phosphorylated peptide Val-Tyr-Thr(P)-His-Glu, which corresponds to fragment 159−163 of cdc2. As expected after bidimensional analysis by TLE and TLC, it was found to migrate exactly as the unique radiolabelled peptide fragment originating from degradation of cyclin A−cdc2 by sequential treatment with trypsin and the V8 protease (Figure 10, panel D). We conclude that cdc2 is phosphorylated on Thr161 in cyclin A−cdc2 complexes. Using a similar approach, we could also confirm that cdc2 is phosphorylated on the same residue in active cyclin B−cdc2 complexes (data not shown).

Discussion

Whilst cyclin proteolysis has been demonstrated to be required for both cyclin B− and cyclin A−cdc2 kinase inactivation, circumstantial evidence exists to support the

view that even after cyclin proteolysis free cdc2 may in some cases still have H1 kinase activity. For example, a major H1 kinase activity was still observed after cyclin proteolysis in cycling *Xenopus* extracts containing unreplicated DNA (Hutchinson *et al.*, 1989). A purified H1 kinase was also prepared from starfish oocytes at first meiotic metaphase, which contained cdc2 as a major protein, however, no associated cyclin was detected by silver staining (Labbé *et al.*, 1988, 1989b). Although it was subsequently shown that the cyclin subunit had undergone proteolysis during the gel filtration step of the long-lasting procedure used in these early experiments to purify cdc2 kinase and that proteolytic fragments of cyclin B were present in the highly purified preparation (Labbé *et al.*, 1989a), it was difficult to escape the conclusion that no stoichiometric association of cyclin with cdc2 was required for the purified protein to maintain a high H1 kinase activity. Finally, the H1 kinase activity of cdc2 was shown not to drop when *cdc13-117*^{ts} mutants at M phase were transferred from the permissive to the restrictive temperature (Moreno *et al.*, 1989), although p56^{cdc13} was reported to dissociate from cdc2 in such conditions (Booher *et al.*, 1989). Taken together these previous reports suggested that some other event was required in addition to the release of a free cdc2 subunit, for the kinase to get inactivated.

Genetic investigation supports the view that activity of type 1 phosphatase is required to inactivate cdc2 kinase and for cells to exit M phase. In *Drosophila*, mutants in one of the four genes encoding type 1 phosphatase (PP187B) die at the larval−pupal boundary with little or no imaginal cell proliferation (Axton *et al.*, 1990). In these mutants, the proportion of neuroblasts at mitosis is 2-fold higher than in wild-type, suggesting a defective regulation of the latter stages of mitosis. The significant increase in mitotic index is due to cells showing extremely overcondensed chromosomes and defective chromosome segregation. In *Aspergillus*, the *bimG11* mutation in type 1 phosphatase prevents anaphase separation of daughter nuclei and causes hyperphosphorylation of mitosis-specific phosphoproteins, also suggesting that cdc2 kinase does not inactivate properly (Doonan and Morris, 1989). In fission yeast, cold-sensitive *dis2* mutants undergo morphologically normal and not delayed chromosome condensation, but sister chromosomes fail to separate at the restrictive temperature (Okhura *et al.*, 1989). High copy number plasmids carrying wild-type *dis2*$^+$ or the suppressor *sds21*$^+$ gene rescue *dis2* mutations. The *dis2*$^+$ and *sds*21$^+$ genes encode products that are highly homologous to each other and to mammalian type 1 phosphatase. Since cdc2 kinase activity remains high at the restrictive temperature in *dis2* mutants and rapidly drops upon transfer to the permissive temperature (Yanagida, 1992), it was suggested that type 1 phosphatase activity might be required for cdc2 kinase inactivation and normal anaphase.

In fission yeast, the phenotype of cells expressing a mutant cdc2 where the Thr167 codon was replaced with a Glu167 codon in an attempt to mimic phosphorylation, suggested that Thr167 dephosphorylation (Thr161 in higher eukaryotes) was important for exit from mitosis. Indeed a strain expressing the mutant could pass through the G_1-S and G_2-M transitions repeatedly. However, cells contained several condensed nuclei and formed multiple septa and mitotic spindles (Ducommun *et al.*, 1991; Gould *et al.*, 1991). In the present work, we provided direct evidence that dephosphorylation of cdc2 on residue Thr161 accompanies cyclin degradation. In addition, we showed that OA prevents cdc2 kinase inactivation following cyclin degradation, when it is used at a concentration sufficient to completely block type 1 phosphatase.

When used at a lower concentration, sufficient to completely suppress type 2A phosphatase but inhibiting type 1 phosphatase by only 50%, OA was found to turn on the cyclin degradation pathway (Lorca *et al.*, 1991a) but it did not prevent cdc2 dephosphorylation after cyclin proteolysis (the present work). This strongly suggests that type 1 phosphatase is sufficient to dephosphorylate cdc2 on Thr161 in frog extracts. Although it fits genetic evidence in *Drosophila*, *Aspergillus* and fission yeast, as well as a previous report that microinjection of specific antibodies directed against type 1 phosphatase strongly delays MPF inactivation and exit from meiotic metaphase in starfish oocytes (Picard *et al.*, 1989), this result does not formally rule out the possibility that besides type 1 phosphatase, type 2A phosphatase (or perhaps another OA-sensitive phosphatase) might catalyse Thr161 dephosphorylation as well. Inhibitor 1, which specifically blocks type 1 phosphatase, was not sufficient to prevent cdc2 dephosphorylation in frog extracts once cyclin had undergone proteolysis. Moreover, type 2A phosphatase has been reported to slowly dephosphorylate cdc2 and inactivate partially purified cyclin B−cdc2 complexes *in vitro* (Gould *et al.*, 1991; Lee *et al.*, 1991; Nigg, personal communication). Finally, treatment of a pig kidney cell line with various concentrations of OA suggested that type 2A rather than type 1 phosphatase could be involved in the transition from metaphase to anaphase (Vandré and Wills, 1992). Although it is difficult from presently available evidence to fully appreciate which of type 1 or type 2A phosphatase plays the major role *in vivo* in inactivating cdc2 at exit from metaphase, we note that in contrast to *dis2* mutants, mutants deleted of *ppa2*, a gene encoding a major type 2A phosphatase in fission yeast, readily exit from metaphase and undergo cytologically normal anaphase (Kinoshita *et al.*, 1991; Yanagida, 1992).

Phosphorylation of Thr161 on cdc2 is believed to be required for either formation or stabilization of cyclin−cdc2 complexes (Ducommun *et al.*, 1991) or alternatively to confer H1 histone kinase activity to such complexes (Solomon *et al*, 1992). Presumably, the extent of Thr161 phosphorylation depends on the balance between activity of the OA-sensitive phosphatase(s) and that of an antagonizing kinase. A kinase phosphorylating Thr161 has been partially purified from frog extracts (Solomon *et al.*, 1992). It does not appear to be cell cycle regulated and uses only cdc2 associated with cyclin as a substrate. This fits our finding that in contrast to cyclin−cdc2, free cdc2 cannot be phosphorylated on Thr161 in either metaphase-arrested eggs or extracts prepared from them. In mitotic cells, the rate at which the phosphate group turns over on Thr161 is limited by phosphatase activity, thus this residue is maximally phosphorylated due to activity of the Thr161 kinase. Once cyclin has been proteolysed, however, cdc2 undergoes net dephosphorylation because the kinase cannot phosphorylate free cdc2. This results in inactivation of H1 kinase activity and exit of cells from mitosis.

By impairing a dephosphorylation event which is normally associated with cyclin degradation, we were able to generate a form of cdc2 free of either cyclin A or B and still active

as H1 kinase. The cyclin subunit is believed to target cdc2 kinases to different compartments in the cell (Pines and Hunter, 1991). In contrast to cyclin B–cdc2 kinase, the still active but cyclin-free cdc2 kinase may fail to bind microtubules or associated proteins, thereby changing microtubule dynamics and spindle organization (Belmont *et al.*, 1990; Verde *et al.*, 1990). This may explain why phosphatase mutants impaired in the process of cdc2 kinase inactivation can enter an anaphase-like state, although they fail to segregate chromosomes properly.

Cytokinesis is highly coordinated with mitosis during the normal cell cycle. This high level of coordination is essential to prevent the cell from dividing before chromosome segregation. There is a close correlation between the metaphase spindle axis and the cleavage plane, although any part of the cell surface can participate in furrow formation if the spindle is displaced during a short period of the cell cycle during which a message for cleavage induction seems to be produced (for review see Inoue, 1981). It appears most likely that the cleavage furrow develops where and when it does by reason of local changes which are themselves dependent on microtubule dynamics. Local changes may include mitosis-specific phosphorylation of both caldesmon, which causes its dissociation from microfilaments (Yamashiro *et al.*, 1990) and myosin light chain kinase (Hosaya *et al.*, 1991), which may play an important role in the regulation of the contractility of the actomyosin system during mitosis, as suggested by the recent finding that *Drosophila* mutants in a gene encoding the regulatory light chain of non-muscle myosin are defective in cytokinesis (Karess *et al.*, 1991). At least for non-muscle caldesmon, the mitosis-specific sites have been mapped and shown to correspond to those phosphorylated *in vitro* by cdc2 kinase (Yamashiro *et al.*, 1990; Mak *et al.*, 1991). The cleavage furrow appears to be established by mid-anaphase, although the basis of the correlation between furrow determination and any particular step in mitosis is unclear. Destruction of mitotic cyclins is believed to occur during metaphase (cyclin A) or at the metaphase–anaphase transition (cyclin B) and at least for cyclin B it is strongly dependent on microtubule integrity (Whitfield *et al.*, 1990; Hunt *et al.*, 1992). This raises the possibility that induction of the furrow may involve a form of cdc2 kinase lacking an associated cyclin, such as the Thr161/167 phosphorylated cdc2 monomer. If cyclin–cdc2 kinases were unable to phosphorylate cleavage-specific targets in the living cell, in contrast to the active monomer, this would actually provide the cell with a mechanism preventing premature furrowing before chromosome segregation. Recent reports that associated cyclins (or absence of associated cyclin?) may specify substrate specificity of cdc2 kinases (Minshull *et al.*, 1990; Luca *et al.*, 1991; Buendia *et al.*, 1992; Lorca *et al.*, 1992; Thomas *et al.*, 1992), as well as the finding that a short but significant delay exists in fission yeast between cyclin B proteolysis and the drop in cdc2 kinase activity (Moreno *et al.*, 1989) would be consistent with such a view.

Materials and methods

Egg extracts

CSF extracts were prepared from unfertilized *Xenopus* eggs as described by Murray and Kirschner (1989). To prepare interphase extracts, dejellied unfertilized eggs were transferred in MMR/2 (50 mM NaCl, 1 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.05 mM Na EGTA, 2.5 mM Na–HEPES pH 7.7) containing 100 μg/ml cycloheximide, and the eggs were electrically activated after 15 min. Extracts were prepared as above 20 min after activation, except that cycloheximide (20 μg/ml) was present throughout the procedure.

cdc2 kinase and recombinant proteins

Starfish cyclin B–cdc2 kinase (MPF) was prepared as previously described (Labbé *et al.*, 1989a, 1991). A full-length human cyclin A cDNA (Wang *et al.*, 1990) was inserted in the expression vector pET3c (Rosenberg *et al.*, 1987). Cyclin A was produced in a soluble form in the *E.coli* BL 21 host induced for 2–3 h at 25°C in the presence of 0.4 mM IPTG.

Full-lengh starfish cyclin B cDNA was cloned in the expression vector pUEXZZB1B2 (Lorca *et al.*, 1992) downstream of the PL λ promotor. It was produced as a fusion protein containing 40 residues of *cro-lac*I sequences at its N-terminus and 219 residues of the serum albumin binding domains of streptococcal protein G- at its C-terminus. Expression of the fusion protein was induced by raising the temperature from 30°C to 42°C in order to inactivate the thermosensitive repressor encoded in the vector. The soluble cyclin B fusion protein was purified from the bacterial extract by binding to human serum albumin (HSA)–Sepharose, followed by elution in HEPES 10 mM pH 7.2 containing 0.25 M lithium diiodosalicylate and desalting on a Sephadex G-25 column.

Chemical and proteolytic cleavages

Cdc2 purified by SDS–PAGE was cleaved by *N*-chlorosuccinimide as described in Lischwe and Ochs (1982). Cyanogen bromide cleavage was carried out according to Gross (1967) in 0.5 ml of a 0.1% solution of cyanogen bromide in 70% formic acid. After an overnight treatment, the gel pieces and the 70% formic acid supernatant were lyophilized and loaded on a second SDS–PAGE gel after the addition of Laemmli sample buffer.

Tryptic digestion was performed in 0.1% (w/v) ammonium bicarbonate with 0.1 mg of TPCK–trypsin (Serva, Heidelberg, Germany) at 37°C. After 16 h, TPCK–trypsin was added a second time. After 8 h the gel piece was removed and the supernatant lyophilized.

The resulting peptide mixture was further digested in 0.1% ammonium bicarbonate by endoproteinase-Glu-C (Boehringer Mannheim) from *Staphylococcus aureus* V8 (0.02 mg) overnight at 37°C. The digestion was stopped by lyophilization. The trypsin plus endoproteinase Glu-C digest was treated by *N*-chlorosuccinimide according to Schechter *et al.* (1976) in a minimum volume of 40 mM NCS in 50% acetic acid.

Phosphoamino acids analysis and peptide mapping

Phosphoamino acids analysis was carried out according to Cooper *et al.* (1983). Phosphopeptide mapping was carried out either by mono-dimensional thin layer electrophoresis (TLE) or by TLE followed by ascending chromatography on cellulose thin layer plates (TLC). Monodimensional electrophoresis was run at pH 1.9 in formic acid–acetic acid–water (50/156/1794, v/v/v) according to Boyle *et al.* (1991). For bidimensional peptide mapping, electrophoresis was at pH 3.5 in pyridine–acetic acid–water (10/100/1890, v/v/v) with migration toward the cathode followed by ascending chromatography right angle in isobutyric acid–*n*-butanol–pyridine–acetic acid–water (1250/38/96/58/558, v/v/v/v/v) as in Boyle *et al.* (1991).

Peptide synthesis

Solid phase synthesis of the Val-Tyr-Thr-His-Glu peptide was performed on a 9050 Milligen Synthetizer with the use of pep Syn KA resin and FMOC as temporary α amino acid protection. Upon completion of the peptide chain assembly the single free hydroxyl group of threonine residue was phosphorylated according to Otvos *et al.* (1989), cleaved from the resin with a TFA–water–phenol mixture and purified further by ionic exchange chromatography on a Mono Q HR 5/5 column eluted with a 0.05 M ammonium bicarbonate linear gradient and lyophilization of the phosphopeptide-containing fractions.

Peptide 9–41 of inhibitor I (Aitken and Cohen, 1982) was synthesized the same way and phosphorylated by protein A kinase using [γ-S]ATP in place of ATP to yield a thiophosphorylated peptide resistant to phosphatases.

Immunoprecipitations, gels and Western blotting

Immunoprecipitations were performed after diluting samples in RIPA buffer (150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM Na glycerophosphate, 0.5% Na deoxycholate, 1% Nonidet NP-40, 10 mM Na phosphate pH 7.6) and immune complexes isolated on protein A–Sepharose. In some experiments protein A–Sepharose beads cross-linked to affinity-purified antibodies were used (Schneider *et al.*, 1982).

Separation of proteins by SDS–PAGE was made using the buffer system of Laemmli (1970) and using 10% polyacrylamide gels (0.27% *bis*acrylamide). 1 mm thick slab gels were run overnight at 70 V (tracking

dye migration ~13 cm). For Western blotting, proteins were transferred either to Immobilon P (Millipore) or nitrocellulose (Schleicher and Schull). Immunoblots were analysed using a chemiluminescent method (Amersham ECL) according to the manufacturers instructions.

^{32}P-labelling and protein kinase activities

For *in vivo* labelling, fully grown *Xenopus* oocytes (stage VI) were manually removed from follicle cells, incubated for 3 h in modified amphibian Ringer (100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES pH 7.8, 0.1 mM EDTA) containing 1 mCi/ml [^{32}P]orthophosphate, then 1 μM progesterone and oocytes were allowed to mature in the continuous presence of ^{32}P. Oocytes were homogenized in 5 vol of buffer A (50 mM Na fluoride, 10 mM EDTA, 10 mM Na pyrophosphate and 40 mM Na β-glycerophosphate pH 7.3). Homogenates were centrifuged for 10 min at 4°C at 12 000 *g* and the supernatant was diluted 2-fold with buffer B (300 mM NaCl, 10 mM Na α-naphthylphosphate, 1 mg/ml soybean trypsin inhibitor, 0.5 mM PMSF, 20 mM Na phenylphosphate, 10 mM Na EDTA, 10 mM Na pyrophosphate, 0.2 mM Na vanadate, 40 μM $ZnCl_2$ in 100 mM Tris pH 7.5). The mixture was clarified by centrifugation at 12 000 *g* for 5 min and immediately used for immunoprecipitation or affinity chromatography on p13^{suc1}–Sepharose beads. *In vitro* labelling of cdc2 was made by adding [γ-^{32}P]ATP in *Xenopus* extracts (200 μCi/30 μl).

To measure H1 histone kinase activities, the material retained on p13^{suc1} beads (or in some experiments the crude homogenate) was incubated for 10 min at 25°C in 10 vol of a reaction mixture containing 1 mg/ml H1 histones (Boehringer Mannheim), 10 mM $MgCl_2$, 200 μM ATP (100 c.p.m./pmol) in 20 mM HEPES pH 7.4. Reactions were stopped by spotting on pieces of P81 cellulose, which were extensively washed, then phosphorylated histones were quantified by scintillation counting. Alternatively aliquots were mixed with 1 vol of Laemmli buffer, boiled, then histones were separated by SDS–PAGE and the extent of phosphorylation monitored by autoradiography.

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